Experimental and natural infection of *Simulium sanchezi* by *Mansonella ozzardi* in the Middle Orinoco region of Venezuela

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Abstract

Experimental and natural infections of *Simulium sanchezi* by *Mansonella ozzardi* were studied in the area of Síquita, Territorio Federal Amazonas, Venezuela. The microfilariae developed synchronously in the blackflies, reaching stage L3 in seven to eight days at temperatures between 23° and 27°C. Larvae in different stages of development, including infective forms, were found in 0·6% of 662 unfed wild-caught females. These results confirm that simuliids are the main vectors of *M. ozzardi* in the American continent.

Introduction

Simulium sanchezi Ramírez-Pérez, Yarzábal & Peterson, 1982** is a blackfly with anthropophilic habits, found in lowland riverine areas of the western part of the Territorio Federal Amazonas (TFA) of Venezuela. Its distribution ranges from Puerto Ayacucho to San Carlos de Río Negro (type locality) (Fig. 1), where its immature stages were collected and reared to adults. The taxonomic study which led to its description was made in March 1982, when 65 adult females were captured in Síquita, a small Goahibo Indian village, which, at the same time, was shown to be an endemic focus of *Mansonella ozzardi* infection (BOTTO *et al.*, 1983). In this area *S. sanchezi* is the most common man-biting dipteran, although ceratopogonids and tabanids sometimes feed on man. The *S. sanchezi* females were dissected and one harboured, in its thoracic muscles, three pre-infective (L2) larvae of *M. ozzardi* (RAMÍREZ-PÉREZ *et al.*, 1982) whose morphology and dimensions (J. Ramírez-Pérez, personal communication) corresponded to those recorded by TIDWELL *et al.* (1980). Since blackflies are known to transmit *M. ozzardi* in Brazil (SHELLEY & SHELLEY, 1976; SHELLEY *et al.*, 1980) and Colombia (TIDWELL *et al.*, 1980), the present study investigated the capacity of *S. sanchezi* to allow development of the larval stages of *M. ozzardi* and to become naturally infected.

Materials and Methods

Study area

The capture and experimental infection of adult *S. sanchezi* females were carried out in Síquita (4°13'N, 67°47'W), a small village on the eastern bank of the Middle Orinoco river, in the Department of Atures (TFA), about 30 min by motor launch north from San Fernando de Atabapo (Fig. 1). The settlement, located at 100 m above sea level, is surrounded by tropical moist forest which borders the river.

Experimental infection

The infection of the simuliids was undertaken in three sessions each of 2·5 hours (from 16.00 to 18.30 hours), on 21, 22 and 31 July 1982. Two volunteers exposed their arms and legs to the bites of the blackflies. The *M. ozzardi* microfilaraemia levels of the volunteers (EC and RY) had been previously determined as 62 mff and 10 mff per 20 mm^3 of blood respectively by the method of KNOTT (1939) using peripheral venous blood (BOTTO *et al.*, 1983). The insects were collected when fully engorged and maintained following the technique described in RAMÍREZ-PÉREZ *et al.* (1976) (which does not include the use of antibiotics in the sugar solution or boiling it).

The simuliids captured during the first two experiments were taken to the field laboratory of C.A.I.C.E.T. at San Fernando de Atabapo. Those collected on July 31 were taken to the INDER entomology laboratory in Villa de Cura (Aragua State, 10°2'N, 67°29'W, 520m). The captive blackflies were examined daily at 8.00, 14.00 and 20.00 hours, any dead specimens being removed and dissected immediately. At the same time, a variable number of live individuals were killed with chloroform and also examined in order to obtain a sufficient number of flies to follow the daily development of the parasite. Each specimen was separated into head, thorax and abdomen under a stereo microscope. These parts were placed into separate drops of saline (0·85% NaCl) on slides, teased with entomological needles, covered with cover-slips and examined for larvae at magnifications of 100 × and 400 ×. When nematode larvae were found they were measured using an ocular micrometer and drawn with the aid of a camera lucida. In some cases, a few drops of 0·03% aqueous methylene blue were added to facilitate morphological studies. Material selected for preservation was fixed with 2% glutaraldehyde in 0·1 M cacodylate buffer. The preparations, mounted in Entellan(R) (Merck), are stored in C.A.I.C.E.T., Puerto Ayacucho. The simuliids were identified by J. Ramírez-Pérez. Some recently killed specimens were mounted and kept as voucher specimens in the Vector Study unit of INDER, Villa de Cura.

Diurnal biting activity and natural infection

In one of the sessions, all the blackflies that landed on the exposed arms and legs of an uninfected volunteer during the first 15 mins of each hour (from 07.00 to 19.00) were collected using a glass aspirator to determine diurnal biting activity. The ambient temperature and humidity during the collection period were recorded. These wild-caught females were stored immediately in 80% ethanol and dissected

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**The description of *S. sanchezi* as a new species by RAMÍREZ-PÉREZ *et al.* (1982) is considered premature by some workers, who prefer to include it as an intraspecific variant of *S. oyapockense* s.l. until chromosomal evidence is available to clarify its status (Dr. A. J. Shelley, personal communication).

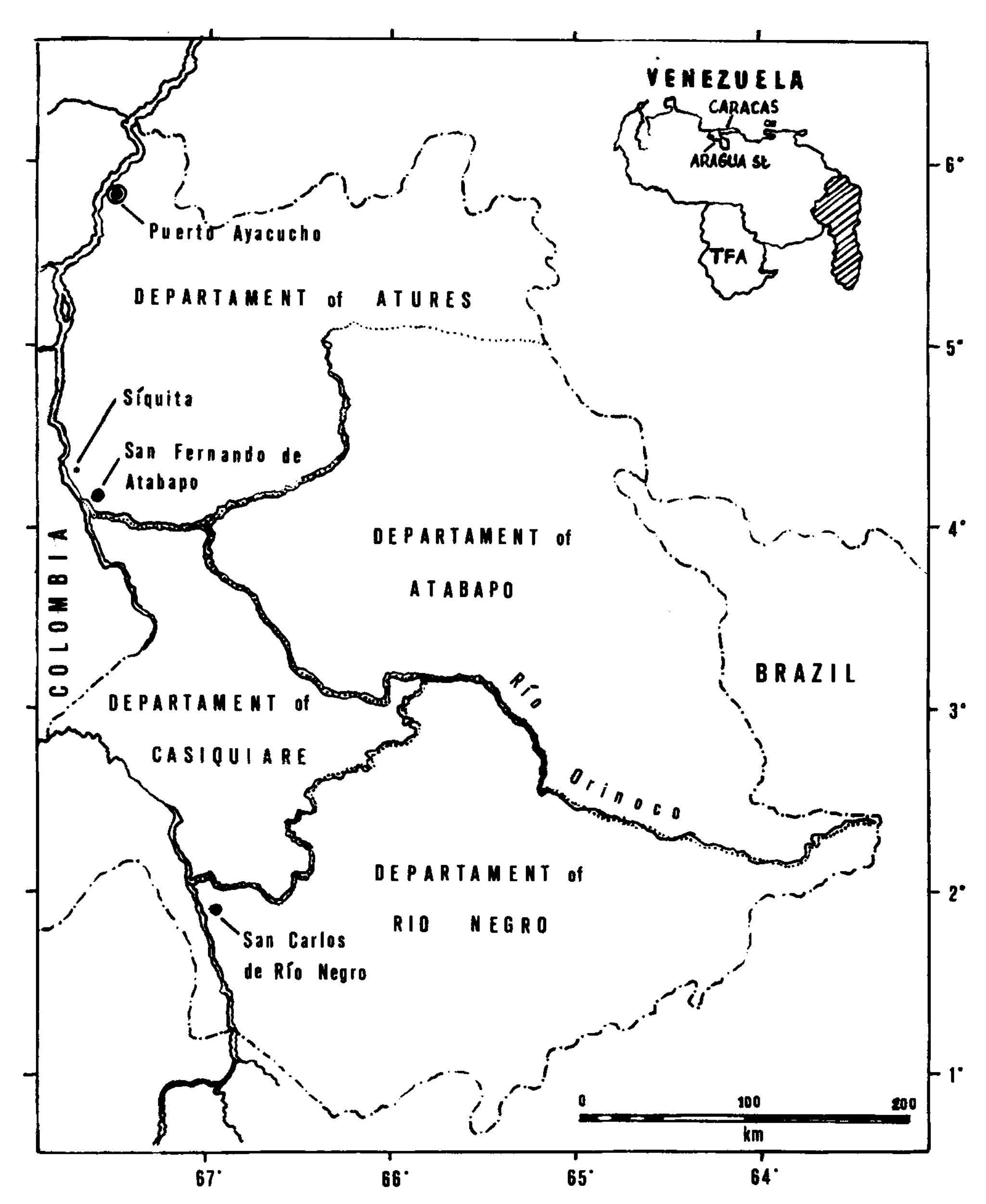


Fig. 1. Map of the Territorio Federal Amazonas (Venezuela) showing the localities mentioned in the text.

subsequently using the technique described by NELSON (1958) to investigate their natural infection rate with *M. ozzardi*.

Results

Experimental infection

During the study periods the temperature in the San Fernando de Atabapo laboratory varied between 23° and 25°C, and between 25° and 27°C in the laboratory of Villa de Cura. The infection rate of the simuliids which took a blood meal from patient EC was 39·6%, in contrast to 25% for those that were fed on RY. The mean number of larvae per infected blackfly was 1·76 and 1·50 respectively. Most flies (83%) were collected from patient EC. The combined data, presented in Table I, show that 37·2% of the insects that engorged on the *M. ozzardi*-infected volunteers were subsequently positive for *M. ozzardi* microfilariae and/or larvae. The total number of parasites found was 90. The parasite load per insect varied between one and six, with a mean of 1·73. Although 59·6% of the infected flies were dead at the time of dissection, 72·2% of the microfilariae and larvae recovered were alive, regardless of the condition of the flies. Microfilariae and larvae were

Table I—Experimental infection of *Simulium sanchezi* with *Mansonella ozzardi* in the area of Síquita, (TFA, Venezuela), July 1982. Flies maintained at 23-27°C

		Time in days after blood-meal											
		0	1	2	3	4	5	6	7	8	9	10	Total
No. flies pos./Total dissected		3/9	2/14	14/29	8/16	3/6	5/16	3/11	7/24	3/8	3/6	1/1	52/140
% Positive		33·3	14·3	48·2	50·0	50·0	31·2	27·3	29·2	37·8	50·0	100·0	37·2
Total no. of *M. ozzardi* larvae		5	6	22	15	5	6	4	13	7	5	2	90
Mean no. larvae per positive fly		1·7	3·0	1·6	1·9	1·7	1·2	1·3	1·9	2·3	1·7	2·0	1·7
% of larval stages	mf	100[a]	100	81·8	13·3	—	—	—	—	—	—	—	
	L1	—	—	13·6	86·7	100·0	66·7	—	—	—	—	—	
	L2	—	—	—	—	—	33·3	100·0	38·5	57·1	—	—	
	L3	—	—	4·6	—	—	—	—	61·5	14·3	100·0[b]	50	

[a] First record of mf in the thoracic muscles
[b] First record of L3 larvae in the head

considered to be alive if their external and internal structures were intact and if they were moving.

31 (35·6%) of the parasites found were in the microfilarial stage at the time of dissection, whereas 56 (64·4%) had undergone developmental changes. 19 (61·3%) of the microfilariae were seen in the abdomen and 13 (38·7%) in the thorax. Only 5 of 23 flies (21·7%) dissected between 0 and 24 hours had ingested microfilariae (mean: 2·2, range: one to three). At the first dissection, four hours after the blood meal, microfilariae were already present in the thorax. Ten (17·8%) of the 56 maturing larvae were located in the abdomen (including L1, L2 and L3 stages), 43 (76·8%) in the thorax and three (5·4%) in the head (all being L3 larvae).

The morphology of the developing larvae closely resembled the description by TIDWELL *et al.* (1980) for *M. ozzardi*. Biometrical data are presented in Table II. The L1 larvae appeared on day 2 but were seen until day 5. Larvae L2 were observed from days 5 to 8. The first L3 larvae were seen during day 7, reaching the head on day 9. The oesophagus occupied 67% of the total body length, the muscular portion comprising 22% and the glandular portion 45%; 19% corresponded to the intestine, 4 to 6% to the rectum and 7 to 8% to the tail. These proportions confirm the identity of these infective larvae as *M. ozzardi*, even though in only 3 of the 16 specimens was it possible to distinguish the four posterior papillae described by TIDWELL *et al.* (1980).

14 of 39 flies (36%) dissected on days 7 to 10 were positive with 56% of the 27 larvae recovered being in the third stage, 33% in the second stage, and 11% not fully determined due to damage during dissection, but recognizable as *M. ozzardi*. The mean L3 load per infective fly was 1·9 (range one to four).

No abnormal forms were seen, but nematode larvae from species other than *M. ozzardi* were observed in 9 (6·4%) of the 140 flies examined, one of them harbouring five mermithids. The presence of larvae from previous blood meals was detected in one fly, in which one *M. ozzardi* L3 form was present with microfilariae in the abdominal cavity on day 2.

Diurnal biting activity and natural infection

S. sanchezi was active throughout the whole day but showed a marked peak of biting activity in the late afternoon, between 17.00 and 18.00 hours. The ambient temperature ranged between 23 and 28°C and the relative humidity between 60 and 81%.

Only four (0·6%) of 662 blackflies collected in Síquita on uninfected humans revealed filarial larvae whose morphological and biometric characteristics coincided with developmental stages of *M. ozzardi*. Three of the flies contained two, six and eight L1 larvae in the thorax respectively (mean: 5·3), and the other had two L3 larvae in the head. This represents an infectivity rate of 0·2%.

Discussion

The results obtained in this study show that microfilariae of *M. ozzardi* are capable of developing to the infective stage after experimental infection of *S. sanchezi* and confirm that this species of blackfly is infected naturally with *M. ozzardi* in the middle reaches of the Orinoco river.

Table II—Biometric measurements of larval stages of *Mansonella ozzardi* in females of *Simulium sanchezi* experimentally infected

Stages	mf	L1		L2	L3
Days post-feeding	0-2 (n = 14)	2-4 (n = 15)	5 (n = 3)	6-8 (n = 9)	7-9 (n = 7)
Total length	191·5 ± 22·9 (135-232)	151·7 ± 18·3 (131-165)	229·6 ± 19·5 (216-243)	372·9 ± 58·7 (307-415)	568·6 ± 65·3 (500-697)
Maximum width	4·4 ± 0·8 (3-6)	14·9 ± 3·2 (12-20)	23·2 ± 1·7 (22-25)	22·9 ± 1·8 (20-27)	19·4 ± 3·5 (18-23)
Position of:					
nerve ring	—	23·3 ± 0·1[a]	36·5 ± 0·0	40·9 ± 4·0 (33-45)	47·5 ± 10·4 (40-55)
excretory cell	—	—	—	55·6 ± 7·7 (45-61)	77·7[b]
Length of:					
oesophagus	—	—	—	225·5 ± 11·1 (216-241)	380·7 ± 3·2 (379-383)
intestine	—	—	—	103·1 ± 26·6 (77-138)	106·4 ± 23·2 (90-123)
rectum	—	—	—	27·6 ± 9·7 (17-40)	24·6 ± 9·8 (18-32)
tail	—	10·7 ± 2·7[c] (6-15)	38·2 ± 4·6[d] (35-42)	37·5 ± 5·2[d] (30-43)	40·7 ± 5·8[d] (35-47)

Measurements: $\bar{x}$ (in µm) ± SD. Range inside parentheses. [a] n = 2; [b] n = 1; [c] measurement from the base to the extreme of the caudal tip; [d] distance from the anal pore to the extreme of the caudal tip.

Table III—Comparison of the cycle of *Mansonella ozzardi* in several species of *Simulium* under different conditions of infection

Studies	Species of *Simulium*	Volunteer microfilaraemia (mf/20mm³)	Total experimental infection rate	Mean larvae per pos. fly	Temperature of cycle (°C)	Days at which most flies had L3 larvae	Measurements of L3 larvae (length and width (µm)
Present study Middle Orinoco river, Venezuela	*S. sanchezi*	EC = 62 RY = 10	39·6 25·0	1·8 1·5	23-27	7-9[a]	568·6 × 19·4 t.l. = 7-8%[b]
TIDWELL *et al.*, 1980. Colombian Vaupés	*Simulium* sp.	I = 128 II = 373	61·0 80·0	2·5 6·8	26-28	6[a]	630·0 × 18·0 t.l. = 7-10%
SHELLEY *et al.*, 1980. Brazilian Amazon	*S. amazonicum* *S. argentiscutum*	112	44·8 69·2	5·3 8·0	19-25	7[a]-9	616·7 × 14·1 t.l. = 7%
TIDWELL & TIDWELL, 1982 Colombian Amazon	*S. amazonicum*	I = 33 II = 63	31·9 44·3	0·7 0·9	24-27	7[a]-9	691·0 705·0
	S. argentiscutum	II = 63	78·5	3·9	23-30	7[a]-8	735·0
NATHAN *et al.*, 1982 Western Guyana	*S. minusculum*	49 75 102	11·8	1·0	Ambient (unspecified)	7	—

[a] days at which L3 larvae were first seen in the head
[b] t.l. = % of the tail length in respect to total body length

Under laboratory conditions the development of the helminth in *S. sanchezi* was synchronous but progressed slowly with L1 larvae being present until day 5, L2 forms until day 8 and the infective stage first appearing on day 7 and reaching the head as late as day 9 after feeding. The observation of L1 and L2 larvae in the abdomen of some flies was probably a result of thoracic muscle fibres remaining with the first abdominal segment during sectioning rather than a true localization of these stages in the abdomen. The possibility exists that some larvae were present from previous infections but the low natural infection rate observed in this study suggests that this was probably insignificant.

The results of our experimental infection are compared with those of other authors in Table III. It is clear that the development of the infection depends closely upon, among other factors, the species of *Simulium*, the level of microfilaraemia in the human population and the temperature at which the cycle takes place. The influence of the blackfly species is exemplified by the greater infection rate found in *S. argentiscutum* when compared with *S. amazonicum* in experiments where other conditions remained constant (SHELLEY *et al.*, 1980; TIDWELL *et al.*, 1982).

There appears to be a correlation between the microfilaraemia of the host and the number of larvae developing in the insect following a blood meal but species-specific factors may also be involved here. Finally the rate of development of larvae in the fly depends on the temperature of maintenance. The retardation in the maturation of L3 larvae in this study, as well as in the experiments of SHELLEY *et al.* (1980), may have been due to lower ambient temperatures than those reported by TIDWELL *et al.* (1980). A slower rate of larval development was found by TIDWELL & TIDWELL (1982) when flies were kept at lower temperatures (22 to 27°C) than those at which the main group were kept (26 to 28°C), or if the temperature was allowed to fluctuate between 23 or 24°C and 30°C.

The natural infection rate found for this July investigation period (rainy season)—0·6% of the sampled population of 0·2% if only flies harbouring infective larvae are considered—is low when compared with those obtained by SHELLEY *et al.* (1980) at the beginning of the rainy season in Brazil; these authors found 3·1% of *S. amazonicum* containing L2 forms and 3·9% of *Simulium* n.sp. containing L3 (*Simulium* n.sp. has since been named *S. argentiscutum* by SHELLEY & LUNA DIAS, 1980). TIDWELL *et al.* (1980) also reported a higher rate for an undescribed species of the *S. sanguineum* group*, with 3·4% of flies being infected with L3 at the end of the dry season in Colombia, but NATHAN *et al.* (1982) observed a low level of naturally acquired infections (0·6% of flies with mature stages) in *S. minusculum* captured in Guyana at the onset of the dry season. The low numbers of flies examined in some of these studies may, however, mean that there is little significant difference between the infection rates reported.

The ability of the "Amazon" strain of *M. ozzardi* to complete its development in *Culicoides* flies, either in wild-caught (TIDWELL & TIDWELL, 1982) or in colonized (LOWRIE *et al.*, 1982) species should be borne in mind. With the exception of the preliminary study by MISRA *et al.* (1952) no efforts have been made to evaluate the real importance of culicoid species in the transmission of filarial parasites in Venezuela. Nevertheless, the evidence accumulated to date indicates strongly that simuliids are the main vectors of *M. ozzardi* in the continental part of America and a more quantitative approach is now needed in the study of the transmission of this disease.

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*The distinction between *S. sanguineum* and *S. amazonicum* groups is now considered to be artificial, all the species referred to in this paper belonging to the *S. amazonicum* group (Dr. A. J. Shelley, personal communication).

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